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EXAMINATION FOR COMPLEMENT-REQUIRING NEUTRALIZING ANTIBODIES AGAINST JAPANESE ENCEPHALITIS, WESTERN EQUINE ENCEPHALITIS AND VACCINIA VIRUSES

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ABSTRACT

Rabbits and guinea pigs were immunized with beta-propiolactone inactivated Western equine encephalitis (WEE) virus vaccine and complement (C') enhancement of early and late neutralizing antibodies was compared. If the early antibody had shown a greater C' enhancement than the late antibody, it would have been regarded as a good tool for early diagnosis of infection. However, contrary to our previous work, the early and late sera showed the same extent of C' enhancement, which was 2 to 4 -fold throughout. The reason for the discrepancy between the earlier and the present results was explored. Since herpes simplex virus induced early neutralizing antibody which was enhanced by C' in an all-or-none fashion but induced a quick development of the late type antibody after a booster injection, it was thought possible that a virus closely related to WEE virus may have infected the animals previously and resulted in a booster effect. However, the anti-WEE rabbit serum did not contain antibody cross-reactive with a very closely related Sindbis virus even in the presence of C'. It may be possible that the previous vaccine contained some viable virus which induced a response different from that induced by inactivated virus. Alternatively, it may be that there are a group of viruses which induce the herpes-type response while other viruses induce the WEE-type response with respect to the extent of C' enhancement of early neutralizing antibody.

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SECTION I

Introduction

Until recently, it had been believed that the viral neutralization did not require complement (C'). Although some workers found a slight enhancement by fresh normal serum of the neutralizing activity of immune serum in certain cases, as summarized in our earlier report (1), it has been a common practice to perform the serum neutralization test for diagnostic purposes without involving C' in the test system. The importance of C' in the neutralization test was first brought forward when we uncovered the fact that the early immune serum of rabbits immunized or infected with herpes simplex virus contained a hitherto unknown antibody which could neutralize virus in the presence of C' (1,2). Later, it was established that this was also the case in guinea pigs and humans (3). Other investigators working with rubella (4), Japanese encephalitis (5,6), vaccinia (7), bovine parainfluenza (8), Newcastle disease (9), respiratory syncytial (10), certain group B arbo (11,12) and SA-8 viruses (13) also revealed the occurrence of C' enhancement of neutralizing antibody.

In the case of early antibody against herpes virus, the addition of C' to the in vitro neutralization system resulted in an all-or-none type reaction. Namely, a serum possessing no or a negligible amount of neutralizing antibody as detected in the absence of C' frequently exhibited a high titer of neutralization in the presence of C'. Late or convalescent sera did not show such a type of reaction but showed only a slight degree of C' enhancement. This fact was confirmed by Hampar et al. (15). On the other hand, however, Heineman (16) could not find the all-or-none type C' enhancement of early neutralizing antibody in sera of adult human patients, and stated that the all-or-none type C' enhancement of early neutralizing antibody might be limited to the primary infection among children.

The above all-or-none type reaction is extremely useful for the early diagnosis of infection, since no other serological means is available at present which can give an unmistakable diagnosis in an early stage of viral infection. Our searches were directed, then, to the

following two subjects. First, it was desired to know whether viruses other than herpes simplex would also induce the complement-requiring neutralizing (CRN) antibody, and if so whether the all-or-none type reaction could be seen in the early serum so that it served for early diagnosis of infection. Secondly, the question raised by Heineman (16) concerning the difference of C' enhancement between the early serum of infant patients and that of adult patients was to be solved.

The greater degree of C' enhancement of early neutralizing antibody as compared to that of late antibody has been discovered not only with herpes virus (1,2,14) but also with vaccinia (7), bovine parainfluenza (8), Newcastle disease (9) and SA-8 viruses (13). In the case of vaccinia virus, however, this was testified only in the immunization of rabbits and such a marked C' enhancement of early serum could not be observed when humans were re-immunized with smallpox vaccine. The difference between animals and humans, as well as the above-cited difference between infant and adult patients suffering from herpetic infection, seemed to reflect a fact that a reinfection might induce a different response as compared with the primary infection with regard to the appearance of CRN antibody.

When we took up Japanese encephalitis virus for the present study, this point was well cogitated. However, it was inevitable that the animals used for immunization were very likely to have been bitten by virus-harboring mosquitoes during the preceding years. Therefore, when our earlier results (17) indicated the absence of the all-or-none type C' enhancement of early sera of immunized rabbits, it was still too early to conclude that the all-or-none type C' enhancement did not occur in the case of Japanese encephalitis virus. In contrast, rabbits immunized with Western equine encephalitis (WEE) virus seemed to show some difference between the C' enhancement of the neutralizing activity of early serum and that of late serum (16). Since this virus is not endemic in Japan, we can exclude the possibility of pre-exposure of experimental animals to the virus. Hence, a more careful examination was undertaken to decide whether this virus could induce an early neutralizing antibody enhancable in an all-or-none fashion.

In parallel with this investigation, it was also studied whether a booster infection to animals after the

disappearance of neutralizing antibody would really induce a different response from that of the primary immunization. For this purpose, the system of herpes simplex virus and rabbits was employed because the serological response in this combination has well been established, as stated above.

SECTION II

Complement (C') Enhancement of Early Neutralizing Antibody Appearing in Animals Immunized with Western Equine Encephalitis (WEE) Virus

1. Materials and methods

a. Viruses. McMillan strain of WEE virus was serially passaged through mouse brains and then through chick embryo (CE) cells. The CE line was cloned once to isolate the small-plaque variant (18). This virus was further passaged through CE cells and used as the test virus for the in vitro neutralization test. A subline of the small-plaque variant passaged through adult mouse brains was used for vaccine preparation. Sindbis virus was maintained by CE cell culture. Quantitation of the viruses was done by plaque counting in CE cells as stated previously (19) and virus titers were expressed as plaque-forming units (PFU).

b. Vaccine. Baby mice of DD/n strain aged 2 to 3 days were infected with an appropriate dilution of the above WEE virus and their brains harvested before death. A 20 % emulsion of the brains was made with buffered saline of pH 7.2 and clarified by centrifugation at 6,000 rpm for 1 hour. Beta-propiolactone (BPL) was dissolved at 1 : 300 in 0.05 M borate-buffered-saline of pH 9 (20) and added to one portion of the above virus emulsion at 10 %. After 3 days' incubation at 4 C, the inactivation was complete. After a further incubation at 37°C for 1 hour, the vaccine was added with Na merthiollate at 10,000 x and stored in an ice box. The rest of the virus emulsion was kept frozen to be used for booster immunization.

c. Immunization of rabbits. Three rabbits weighing about 3 kg which were free of nonspecific virus-

inhibitors were given the vaccine intravenously in two doses of 0.5 ml with an interval of 1 week. The vaccine contained 4 x 10^9 PFU/ml of BPL-inactivated virus. One week after the second injection, live virus was given subcutaneously in the amount of 0.5 ml as a booster. Bleeding was done before immunization, and 3 days and 1, 2, 3, 4, 6 and 8 weeks following the first injection.

d. Immunization of guinea pigs. Guinea pigs which were free of nonspecific virus-inhibitors were immunized once by intraperitoneal injection of 0.5 ml of a vaccine which contained 6.2 x 10^9 PFU/ml of BPL-inactivated virus. Bleeding was performed according to a similar schedule as in the immunization of rabbits, each time selecting 4.

e. Antisera. The sera separated were inactivated by heating at 56°C for 30 minutes, and stored in a deep freezer before and between tests.

f. Magnesium saline (MgS). The diluent for serum and C' was the following magnesium saline (MgS). Physiological saline containing 0.01 % $MgCl_2$ was first adjusted to neutral pH by the addition of NaOH and then Tris-HCl buffer of pH 7.8 was added to the final concentration of 0.0025 M. It was autoclaved, rubber-stoppered and stored in an ice box. Each bottle was opened but once, and any volume remaining after a test was discarded.

g. Neutralization test. The agar cover-slip (ACS) technique for inoculation of virus-serum mixtures (21) was improved by the use of Millipore-filtered seed virus which was prepared by the method of Wallis et al. (22), as stated previously (17). The CE line of WEE virus small-plaque variant was centrifuged at 5,000 rpm for 1 hour and passed through a Millipore filter of 0.1 μ porosity which had been coated with 10 % fetal calf serum. The filtrate was added with inhibitor-free calf serum at 10 %, distributed in glass ampoules, shell-frozen in an acetone-dry ice mixture and kept at -70°C. One ampoule was immediately opened for determination of the PFU titer. At each test, 2 ampoules were thawed, pooled and diluted with physiological saline containing 20 % normal calf serum to a strength of 1 x 10^7 PFU/ml. Each test serum was diluted by 2-fold increments with MgS in series of 0.2 ml each. Control tubes received 0.2 ml of MgS. One series received 0.1 ml of 10 hemolytic units of C' as determined by the method described earlier (2), while the other series received the same amount of MgS,

care being taken to keep the tubes at an ice-cold temperature. Then all tubes received 0.1 ml of the above virus. After an incubation at 37°C in a water-bath for 1 hour, the mixtures were cooled, and inoculated to CE cells by the ACS technique (21). The inoculated CE cell dishes were incubated at 35°C for 1 day, and stained with 1 : 10,000 neutral red agar as performed before (21). Control mixtures formed confluent plaques under the ACS, and the highest serum dilution resulting in reduction of infectivity as judged by the formation of no or scattered plaques was taken as the endpoint. The neutralization titer was expressed by the reciprocal of the endpoint dilution.

2. Results

A large part of effort in the present study was directed to the improvement of the neutralization test procedure, as a continuation from the previous work (19, 21). Eventually, it was found that when the seed virus was filtered through a Millipore filter of 0.1μ porosity the unneutralizable persistent fraction was reduced and the reading of results in the ACS neutralization test was easy, because in this test the positive neutralization was judged by reduction of virus infectivity from an amount enough to form confluent plaques down to an amount to from scattered or no plaques. As a result, the procedure previously employed, i.e. an overnight incubation of virus-serum mixtures in an ice box could be obviated and only the incubation at 37°C for 1 hour sufficed.

One trouble which occurred during the present study was that the BPL inactivation of WEE virus often resulted in a poor potency of vaccine. In fact, two trials performed earlier showed that no neutralizing antibody appeared in rabbits after immunization with the vaccine. Later, it was learned that the cause was the use of distilled water in the step of BPL dilution. As stated by Work (20), the antigenicity of arboviruses was labile to acids produced by the BPL treatment. Then we employed borate-buffered-saline of pH 9 (20) at this step.

Table 1 shows the result of the immunization of rabbits with the WEE vaccine. The vaccine was given at days 0 and 7, and a booster with live virus was done at day 14. With all serum samples, an enhancement of the endpoint by C' was observed which was 2 to 4-fold in

most cases. However, contrary to our previous finding (19), there was no stage of immune response showing the all-or-none type C' enhancement of neutralizing antibody. For example, in rabbit no. 1, CRN antibody appeared already at day 3 with a titer of 1 : 20 but at this stage non-complement-requiring neutralizing (non-CRN) antibody was present at a titer of 1 : 5. When CRN antibody rose to 1 : 160 at 1 week, non-CRN antibody was as high as 1 : 40, and thus the difference between the two types of antibody was unaltered from the early to the late stage.

Table 1. Development of CRN and non-CRN antibodies in rabbits immunized with WEE virus.

Rabbit No.	C'	Time after the 1st injection							
		0	3d	1w	2w	3w	4w	6w	8w
1	+	<5	20	160	320	320	320	80	10
	-	<5	5	40	160	80	80	10	<10
2	+	<5	5	20	160	40	20	5	5
	-	<5	<5	5	80	10	5	5	<5
3	+	<5	5	20	320	640	320	80	80
	-	<5	<5	10	320	160	80	40	40

Our previous experiment, which detected a marked C' enhancement of neutralizing antibody in early sera, used guinea pigs. Hence, the discrepancy between the previous and present results was thought to be due to the use of different animal species. Therefore, guinea pigs were then immunized. This time, the vaccine was given intraperitoneally only once. Bleeding was done with a schedule shown in Table 2. Again, the early sera showed a similar response as observed in rabbits with respect to the C' enhancement of neutralizing antibody.

As stated in the next section of the present report, a booster immunization may induce a response different from the primary immunization. We then considered a possibility that, although WEE virus does not

Table 2. Development of CRN and non-CRN antibodies in guinea pigs immunized with WEE virus

Guinea pig No.	C'	Time after the 1st injection				
		0	3d	1w	2w	4w
1	+	<5	<5		80	
	−	<5	<5		10	
2	+	<5	<5		160	
	−	<5	<5		40	
3	+	<5	<5			10
	−	<5	<5			<5
4	+	<5	<5			40
	−	<5	<5			10
5	+	<5		<5		
	−	<5		<5		
6	+	<5		<5		20
	−	<5		<5		5
7		<5		40		20
		<5		5		<5
8	+	<5		80		
	−	<5		10		
9	+	<5			20	
	−	<5			<5	
10	+	<5			20	
	−	<5			<5	

cause an endemic among experimental animals in Japan, some closely related virus exists in nature and pre-exposure to that virus may be the cause for the occurrence of the booster type response in the primary immunization. If so, the C' enhanced neutralization might reveal a cross reaction with other closely related viruses, too, because such a cross was noted among very closely related group B arboviruses (11,12).

To test this possibility, we selected Sindbis virus. This virus is known to be closely related to WEE virus, and there is a one-way cross neutralization between the

two viruses, the anti-Sindbis serum being capable of neutralizing WEE virus even in the absence of C' (23).

The 1-week serum of the above rabbit No. 1 was serially diluted in 4 series. Two series were given C' while the rest received MgS. One C' series and one MgS series were given WEE virus and the other two series Sindbis virus, the amount of viruses being so diluted that the controls contained about 30 PFU per 0.05 ml. After one hour's incubation at 37°C, each mixture was titrated for infectivity. As shown in Table 3, no neutralization occurred with Sindbis virus.

Table 3. Absence of cross neutralization between an anti-WEE rabbit serum and Sindbis virus in the presence or absence of C'

Virus	C'	Number of plaques per 0.05 ml after mixed with the indicated dilution of the antiserum							
		10	20	40	80	160	320	640	Cr
WEE	+	0*	1	1	3	13	27	31	48
	−	2	2	3	11	29	37	36	30
Sindbis	+	26	39	26	26	34	30	34	28
	−	28	33	35	33	29	26	31	32

* Average of 3 parallel dishes.

3. Discussion

The present result is contrary to the result obtained earlier (19) in that neither rabbits nor guinea pigs exhibited an all-or-none type C' enhancement of neutralizing antibody in early serum. This discrepancy of data is to be explored. One possibility is that the animals used had been pre-exposed to some closely related virus previously, and this brought about a booster effect in the present immunization. This was denied, because even the very closely related Sindbis virus could not be neutralized by the C'-enhancable

portion of the rabbit serum.

Another possibility is that the all-or-none type response can be induced by live virus whereas CRN antibody is not provoked to such an extent by inactive virus, and the vaccine used in our previous work (19) did contain much active virus due to an inadequate inactivation. In fact, the presence of viable virus in a killed vaccine is sometimes difficult to detect because of the interfering effect of the inactivated virus with the remaining live virus. The previous vaccine was prepared with BPL dissolved in distilled water, but in our present experience BPL deteriorates viral antigenicity unless dissolved with pH 9 borate-buffered-saline. It is conceivable, therefore, that the lot of BPL previously used was of a poor virus-inactivating activity and left much viable virus, which was not detected by the viability test and which induced a different immune response upon immunization. Although WEE virus is highly pathogenic to guinea pigs even when given parenterally, the presence of a large amount of inactivated virus may have interfered with the progress of fatal infection.

Another possibility may be that, some viruses such as SA-8 (13), vaccinia (7), bovine parainfluenza (8) and NDV (9) cause an immune response similar to that induced by herpes simplex virus (1,2) in that the majority of early neutralizing antibody is CRN antibody, whereas other viruses induce a different response, the early and late sera being enhanced by C' to the same extent. If this was correct, rubella (4), WEE and, perhaps, Japanese encephalitis viruses may belong to one group opposite to the above viruses. Which of these possibilities is correct will be determined in future studies.

SECTION III

Effect of Booster Injection of Herpes Simplex Virus upon the Appearance of Complement-Requiring Neutralizing (CRN) Antibody

1. Materials and method

Materials and methods were essentially the

same as stated before (1,2,3). Ten rabbits were immunized by intravenous injections of live herpes simplex virus, HF strain cultured in HeLa cells, with one-week intervals. Four injections were done, each time giving about 5 x 10^6 PFU of virus as determined by the plaque counting in CE cells. One-and-half years later, three rabbits showed reduction of neutralizing antibody to low levels, and these rabbits were used for the booster experiment. One booster injection was given as above, and bleeding was done 3 days, 1, 2 and 4 weeks later. Two new rabbits received the same virus as control and bled with the same schedule. Serum neutralization tests were performed as in the previous work (2).

2. Results

Table 4 indicates the primary response of the 3 rabbits. The all-or-none type C' enhancement of early neutralizing antibody was clearly demonstrated. After the booster injection, the response was quite different as seen in Table 5.

Table 4. Development of CRN and non-CRN antibodies in the 3 rabbits at the primary course of immunization

Rabbit No.	C'	Time after the 1st injection				
		0	1w	2w	3w	4w
3	+	<5	10	1280	640	640
	−	<5	<5	10	20	40
5	+	<5	5	320	1280	640
	−	<5	<5	10	40	40
8	+	<5	<5	320	320	1280
	−	<5	<5	5	40	40

While the two rabbits of the control group showed similar responses as observed one-and-half years before with these 3 rabbits, the sera obtained after the booster injection no more exhibited such an all-or-none type C' enhancement. In the first place, the develop-

ment of CRN as well as non-CRN antibody was quick and already at the end of 1 week a high titer of neutralizing antibody appeared, the enhancement by C' being 2 to 4-fold. Only at the very early stage, i.e. 3 days after the booster, C' enhancement of 8 to 32-fold was discerned but these serum samples did contain non-CRN antibody at the titer of 1 : 20. There was no stage in which the all-or-none type response, such as seen in the control rabbits, could be detected.

3. Discussion

When Heineman (16) indicated the absence of the all-or-none type C' enhancement of early neutralizing antibody in human patients suffering from herpes virus infections, he used the term of "primary infections of adults". However, it is questionable whether such patients had never been infected by this virus before.

Table 5. Development of CRN and non-CRN antibodies in the booster and control groups of rabbits

Rabbit No.	C'	Time after the booster injection					
		0	3d	1w	2w	4w	6w
3	+	20	320	640	320	320	320
	−	5	20	160	160	160	80
5	+	40	160	2560	2560	320	320
	−	5	20	640	640	80	80
8	+	10	640	640	320	160	160
	−	5	20	160	80	40	20
Control 1	+	<5	10	160	160	80	40
	−	<5	<5	<5	10	20	40
Control 2	+	<5	40	160	160	320	160
	−	<5	<5	<5	10	20	20

The present result may suggest that most, if not all, of the adult patients examined by Heineman may have been attacked by the virus as re-infection. In this connec-

tion, it is important when testing the type of neutralizing antibody in early serum of animals or humans with respect to C' enhancement to know whether the serum antibody expresses the primary or booster type response.

SECTION IV

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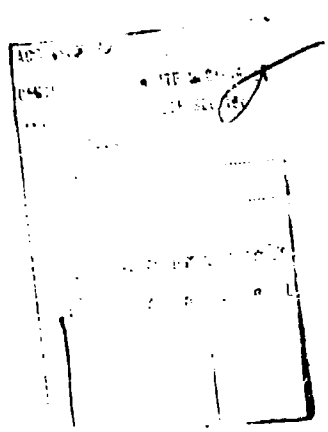

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13. ABSTRACT

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Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Antibodies						
Rabbits						
Guinea pigs						
Virus Diseases						
Japanese Encephalitis						
Western Equine Encephalitis						
Vaccinia viruses						
Tests results						
Cells						
Injection						
Immunization						
Japan						